Vertical Transmission Dynamics of Pea Aphid Symbionts in Natural Settings

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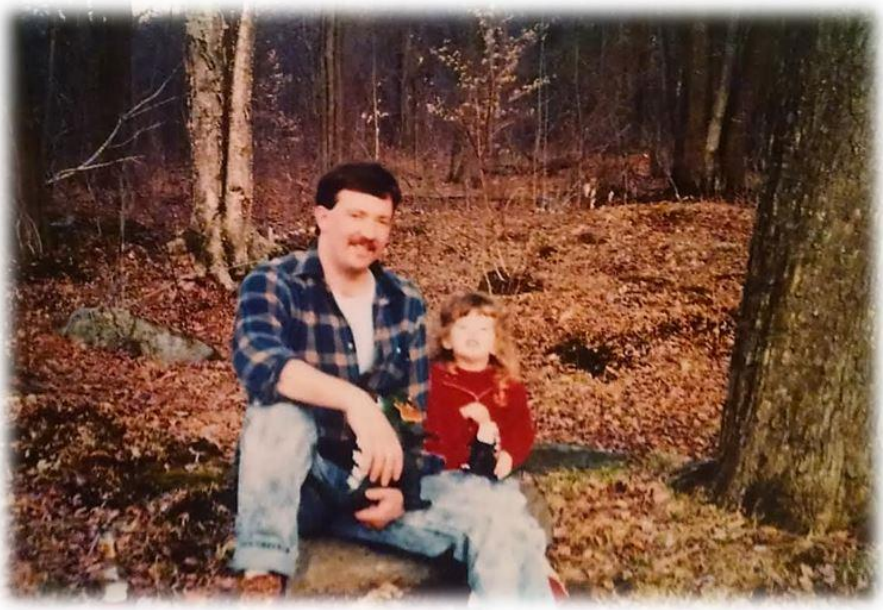
Dedication

In memory of my father, whose curiosity and deep appreciation for nature inspired me to complete my MS in Environmental Science. His unconditional love, guidance and support have stayed with me; and continue to give me the strength and confidence I need to tackle life's most difficult challenges.



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Abstract

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Several bacterial lineages contain members that are known only as symbionts of insects. The pea aphid (*Acyrthosiphon pisum*) harbors eight such bacterial symbionts, some of which have been shown to mediate interactions between aphids and natural enemies like parasitoids, predators and pathogens. These symbiotic relationships persist primarily through maternal transmission, with prior lab-based estimates suggesting near perfect passage of symbionts from mothers to offspring. Yet studies in other systems have noted imperfect maternal transfer of bacterial symbionts, with factors such as temperature and the presence of co-infecting microbes playing a role in this fidelity. Since the prevalence of symbionts in natural host populations will depend on their transmission efficiencies and effects on the hosts' fitness, it is important to understand transmission in a more natural context. In the pea aphid system, transmission rates could conceivably vary between symbiont species, across seasons, or based on the presence of co-infecting symbiont species. This would have implications for the known seasonal dynamics of these symbionts and their overall prevalence in aphid populations. In this study, we performed field sampling, lab rearing and extensive PCR screening to help identify transmission efficiency of pea aphid symbionts. Observations indicate imperfect maternal transfer in the field. While we find no strong evidence for an impact of temperature on transmission rates, the identities of co-infecting symbionts have a large impact on the efficiency of maternal transfer. Interestingly, symbionts living together

often in the field appeared to improve each others' transmission upon coinfection. In contrast, pairings of rare symbiont partners were associated with reduced rates of transfer. In particular, results show statistically significant differences in transmission rates for *Rickettsiella* and *Serratia* inhabiting a pea aphid with and without the presence of *Serratia* and *Rickettsiella*, respectively. Evidence also suggests the presence of *Spiroplasma* in pea aphid microbiomes decreases transmission efficiency of other pea aphid facultative endosymbionts. Given the roles of these symbionts in aphid defense, and the frequent occurrence of inherited, defensive symbionts across plants and invertebrates, our findings on natural symbiont transmission dynamics in pea aphids could have broader implications of importance for many economically, medically, and agriculturally important organisms.

Introduction

Symbiosis is a relationship between two species that are living in close proximity and have direct contact with each other. In many symbiotic relationships, a microscopic organism inhabits a larger eukaryote. These relationships often benefit one or both of the species involved, and can influence their evolutionary trajectory (McFall-Ngai *et al*. 2013). In fact, many significant adaptations in eukaryotes such as aerobic respiration, autotrophy, nitrogen fixation, and the ability to feed on diets low in nutrients have developed through their symbiotic relationships with microbial organisms (Liu *et al*. 2012; Oakley *et al*. 2016; Hansen & Moran, 2014; Russell *et al*. 2009). In many such cases, the relationship between eukaryote hosts and their resident microbes are obligate, or required, for survival by both partners. There is compelling evidence that even mitochondria and chloroplasts were once primitive free-living bacteria cells, which became incorporated into eukaryotic cells over time as the result of a longstanding obligatory symbiosis (Whatley *et al*. 1979).

Not all symbionts are obligatory, and those not required for host growth, development, or reproduction are referred to as facultative. Despite the fact that organisms can live without them, the addition of facultative microbial symbionts can significantly enhance host survival and fecundity (Hrček *et al*. 2016). Mutualistic effects such as defense against natural enemies and resistance to extreme environmental conditions can give organisms a phenotypic advantage, which can help facilitate the colonization of new niches, and may lead to speciation (Simon *et al*. 2003; Tsuchida *et al*. 2004; Lipnicki, 2015). When this occurs, the symbiont and host may develop an

obligatory relationship over time. Pérez-Brocal *et al.* (2006) have seen evidence of a possible shift from facultative to obligatory symbiosis in an aphid symbiont. Their research shows the microbial symbiont, *Serratia symbiotica*, may be evolving into an obligatory symbiont in a group of conifer aphids from the genus *Cinara*. Evidence suggests that *S. symbiotica* may have taken over some roles of the obligate *Buchnera aphidicola* symbiont, cementing its place as a required, co-obligate symbiont that allows these aphids continued utilization of low-nutrient, phloem sap diets. As these shifts take place over evolutionary time within complex systems, determining the exact mechanisms and processes that lead to fixation can be difficult.

The transmission of symbionts between organisms and across generations can have significant impacts on the likelihood of symbionts reaching fixation within a population (Gundel *et al*. 2011). Transmission rates have been shown to be imperfect in natural populations (Jaenike *et al*. 2010) and transmission efficiency could be affected by temporal shifts in symbiont densities caused by environmental factors (Su *et al*. 2014; Burke *et al*. 2010). Interactions between microbial communities living within the host may also be of particular importance to transmission efficiency (Wu *et al*. 2006). There is some evidence that these microbial community dynamics may help explain why beneficial symbionts are not transmitted with complete efficiency, and could significantly influence the likelihood that a symbiont becomes obligatory or remains facultative over time (Vautrin *et al*. 2008). Although it can be difficult to study processes that develop over an evolutionary timescale, the pea-aphid system shows great potential in this effort as their symbionts and symbiont benefits are well-established. Understanding the

dynamics of symbiont transmission efficiency in pea aphids will help us understand how the role of symbionts may evolve over time.

Maintenance of Microbial Symbionts

Mechanisms governing the spread and maintenance of facultative symbionts are comparable to the mechanisms governing the spread and maintenance of beneficial nuclear mutations in adaptive evolution; however, there are some key differences. One key difference is the origin and magnitude of the phenotypic change. In adaptive evolution by nuclear mutation, mutations originate by chance, and are not necessarily correlated with potentially beneficial phenotypic changes (Luria & Delbrück, 1943). Even those few nucleotide changes that do become fixed within a population likely have only minute benefits to the organisms' survival, fecundity, or life history traits. In many instances of microbial symbiosis, hosts commonly acquire new symbionts from similar host organisms as a result of living in close proximity to them. Similar sympatric host organisms experiencing the same selective pressures may benefit from the same types of microbial symbionts (Jaenike, 2012). Hence, the microbial symbionts are more likely to be acquired by multiple host species, and therefore bring with them a set of pre-tested megamutations that can offer a multitude of immediate benefits to the host. (Jiggins & Hurst, 2011).

Symbionts have been shown to provide their hosts with new traits such as enhanced drought tolerance (Clay & Schardl, 2002), the ability to survive in extreme heat (Russell & Moran, 2006), protection against fungal pathogens (Łukasik, 2013), and the

ability to feed on new diets (Hosokawa *et al*. 2007). Since symbionts are more likely to cause significant changes, their acquisition by a host can be referred to as a megamutation (Haynes, 1991). If the symbiont is established in a host population long enough, coevolution could eventually lead to superior fitness within that host species (Bracewell & Six, 2015). The acquisition of these microbial megamutations can have such significant effects upon the hosts' evolutionary trajectory that they become obligate, and as a result they may come to exhibit congruent phylogenies with their hosts (Jaenike, 2012). Phylogenetic reconstructions have revealed host-symbiont associations that have persisted unbroken for hundreds of millions of years. Analysis of 16S rRNA genes has identified symbiont lineages belonging to the phylum *Bacteriodetes* with phylogenies congruent with the phylogenies of sap-feeding insects in the suborder Auchenorrhyncha. Results indicate the shared ancestor of these insects acquired the symbiont at least 260 million years ago (Moran *et al*. 2005).

We do not see the same congruence among phylogenies of facultative symbionts and their hosts (Werren *et al*. 1995). These facultative symbionts are not essential for host survival, but can have a significant impact on insects' life history traits due to the megamutation effect. Despite the high potential for benefits, symbiont frequencies within a host species can vary greatly over time (Aukema, 2005). Pertinent to these dynamics is the observation that facultative symbionts may impose costs upon their hosts, which could occasionally offset benefits depending on the prevailing environmental conditions (Oliver *et al*. 2014). The balance of costs and benefits to the host species affects the persistence of facultative symbionts within the host population (Rudgers *et al*. 2012).

Facultative endosymbionts' impact on host reproduction and survival can dictate the microbes' abilities to persist in a population since success of symbionts depends on their transfer to the next generation of the host organisms. Symbionts are initially acquired by a new host species through horizontal transfer (Russell *et al*. 2003; Baldo *et al*. 2008; Gehrer & Vorburger, 2012). After the initial horizontal transfer, the persistence of a symbiont within a host species is determined by successful transmission of symbionts from mother to offspring. Observations have shown that immediately following a host-shift event symbionts can perform poorly, occasionally exhibiting low rates of vertical transfer (Clancy & Hoffmann, 1997; Russell & Moran, 2005). Successful vertical transmission of the symbiont also necessitates that the host mother survives long enough to pass on the symbiont to its offspring. Thus, the extent to which the newly acquired symbiont aids in the survival of the female host is directly related to the persistence of this symbiont within a host population. Some of the most common ways in which symbionts improve host survival (and other aspects of fitness) include providing protection against parasitoids (Oliver *et al*. 2003), pathogenic fungi (Łukasik *et al.* 2013) and parasites (Jaenike *et al*. 2010). Symbionts can also increase their hosts' ability to utilize novel plants (Tsuchida *et al*. 2004), improve fecundity (Himler *et al*. 2011), and provide a greater tolerance of heat stress (Clay & Schardl, 2002; Russell & Moran, 2006). However, the benefits of harboring bacteria are often coupled with costs and thus a net positive impact of the symbiosis is critical for the symbiont to persist in a host population.

For example, the perennial grass, stout wood reed (*Cinna arundinacea*), is often symbiotic with the endophyte *Neotyphodium schardlii*. *N. schardlii* strongly reduces *C.*

arundinacea survival, but it also increases regeneration and is persistent with 100% frequency because the beneficial effects of regeneration overwhelm the negative effects on plant survival (Rudgers et al. 2012). In situations such as this, where the facultative symbiont's benefits outweigh the cost of harboring it, there may be a higher likelihood of dependence on this symbiont throughout a host population. However, most facultative symbionts with a net positive effect fall short of becoming 100% persistent in a population (Dykstra *et al*. 2014; Haselkorn & Jaenike, 2015). In addition to baseline costs, inefficient vertical transmission is also a driver of sub-100% frequencies within a host population (Gundel *et al*. 2011).

Vertical Transmission Dynamics

The two routes for potential symbiont spread are horizontal and vertical transfer. Horizontal transmission of microbial symbionts can occur through shared feeding sites (Caspi-Fluger *et al*. 2012). Symbionts can also be carried from one insect species to another by biological agents such as mites and other parasites (Jaenike *et al*. 2007), transferred through open wounds (Rigaud & Juchault, 1995), or spread by contaminated ovipositors of parasitoids (Gehrer & Vorburger, 2012). Vertical transmission is transfer from mother to offspring that frequently occurs through the cytoplasm of the eggs or to developing embryos in the case of live-birthing invertebrates. Variation in success rates of vertical transmission may influence variable symbiont frequency within a host species. Even if symbionts have strong mutualistic effects, imperfect transmission rates can reduce symbiont presence within a host population, which means transmission could be a determining factor of the frequency of symbiosis in host populations and the probability

that a symbiont will become a fixed component of the holobiont (Gundel *et al*. 2011; Douglas, 2008).

Vertical transmission has been experimentally shown to help hosts select for symbiotic partners that impose a low cost compared to the benefits provided (Douglas, 2008). Bull *et al*. (1991) conducted an experiment using two strains of the filamentous F_1phage, a bacterial virus of *Escherichia coli*. These two strains of F_1 phage differed in virulence and both were able to pass to new hosts either horizontally or vertically. If allowed to act freely, both horizontal and vertical transmission will occur simultaneously and there will not be a difference in the prevalence of either phage strain. Bull *et al*. 1991 found that if horizontal transmission of these strains was restricted, the less virulent strain became dominant in the population. These findings suggest vertical transmission can assist in selecting for symbiotic partners with traits that enhance performance. This result has been replicated in several systems including endophytic fungi of grasses (Clay & Schardl, 2002) and symbiotic algae *Symbiodinium* in the jellyfish *Cassiopeia xamachana* (Sachs & Wilcox, 2006). These studies raise the possibility that vertical transmission may help protect mutualistic host-symbiont pairings while limiting the spread of costly symbionts. This can be a valuable trait for host organisms, especially when the costs and benefits derived from the symbiont become context-dependent. However, there is still considerable uncertainty about the rates and modes of vertical transmission.

Symbiont density within a host most likely influences the efficiency of vertical transfer. Effects upon symbiont density include environmental factors and microbial community dynamics. Examples of environmental influence on symbiont density have been found in the whitefly species *Bemisia tabaci*. *B. tabaci* contains symbionts whose

densities are impacted by biotype, host plant, geographic location, and low temperatures (Pan *et al*. 2011; Su *et al*. 2014). Researchers have also seen extreme heat lower the density of *Serratia symbiotica* in pea aphids to very low levels and in some cases have cured pea aphids of *S. symbiotica* using heat treatments (Burke *et al*. 2010).

Microbial community dynamics can also play a role in symbiont densities. It is common for multiple symbionts to coexist within individual hosts (Smith *et al*. 2015). When this occurs, hosts are said to have superinfections. Fluctuation of superinfection stability can be impacted by symbiont-symbiont interactions (Douglas, 2008). Hosts have limited shared space and resources available for symbionts. This can lead to competition among symbionts within the host and may even result in symbiont alliances developing over time. Research has shown that microbial communities that have been paired together through vertical transmission for long periods can develop the ability to collaborate and sabotage horizontally transmitted symbionts (Vautrin & Vavre, 2009). This suggests that the community of microbial organisms present in the host could affect successful vertical transmission of individual symbionts.

Studies conducted in laboratories report near perfect rates of transmission (Chen & Purcell, 1997). However, there is evidence for symbiont loss caused by environmental factors (Pan *et al*. 2011; Burke *et al*. 2010) and microbial community dynamics (Douglas, 2008; Vautrin & Vavre, 2009). To gain a better understanding of vertical transmission rates' effect on symbiont persistence and frequency in host populations, it is critical to conduct experiments in natural settings. Making observations in natural settings will help us better predict host-symbiont interaction dynamics and the frequency of symbiosis in nature. One effective way to do this is to gather quantitative vertical transmission data

from a model host species, which can then be used to predict transmission dynamics for this specific organism along with transmission dynamics for hereditary symbionts more generally (Gundel *et al*. 2011).

The Pea Aphid System

Pea aphids (*Acyrthosiphon pisum*) and their bacterial symbionts are an ideal model system for testing hypotheses about the dynamics and frequency of symbiosis. They are cosmopolitan in distribution and are phenotypically variable, which allows for a wide variety of influences of microbial symbionts on host phenotypes. Hence, the combined expression of symbiont and host genotypes may generate a range of phenotypic diversity on which selection can operate and influence host population ecology and evolution (Leclair et al. 2016). Pea aphids are cyclically parthenogenetic in temperate regions and typically have more than eight clonal generations before turning sexual and laying eggs for overwintering (Markkula, 1963). Studying vertical transmission in an organism that reproduces asexually is preferred because the combination of genetic material and shared symbionts that arises from sexual reproduction can complicate the process of hereditary symbiosis. Using a host organism that makes exact genetic copies of itself allows researchers to focus solely on the transmission efficiency of symbionts and the potential differences in transmission efficiency that may be caused by environmental conditions, the type of symbiont, and the combination of symbionts found in the aphid.

Pea aphids are also ideal model organisms for studying vertical transmission dynamics of facultative symbionts in a natural setting because maternal vertical

transmission is thought to be the main pipeline for pea aphids maintaining their facultative symbionts (O'Neill *et al*. 1997). Pea aphids only harbor seven different facultative symbionts and one obligatory symbiont. Hence, there are a manageable number of combinations of facultative symbiont groupings, which allows us to observe differences in transmission rates by comparing and contrasting aphid infections with and without specific facultative symbionts and/or symbiont pairings.

The pea aphid's obligatory symbiont is call *Buchnera aphidicola*. *B. aphidicola* is found in the bacteriocyctes of the aphid (Douglas, 1989). Without *B. aphidicola*, pea aphids cannot obtain the nutrients they need from the plant sap they feed on (Douglas, 1998). This host-symbiont association is estimated to have persisted unbroken for 150-250 million years (Moran *et al*. 1993; Moran & Telang, 1998). On the other hand, the secondary symbionts in the aphid have little phylogenetic congruence with the aphid (Russell *et al*. 2003). These infections are generally short-lived in evolutionary timescales as the symbiont frequencies fluctuate in natural settings (Smith *et al.* 2015). The roles of six of the seven secondary symbionts found in aphids have been determined with relative certainty. These benefits include helping the aphid survive in high temperatures, and defending them against parasitoids and pathogens. It is possible that environmental factors might contribute to the frequency of symbionts in a population. It is also possible that the specific roles of the symbionts might influence the coinfections that occur naturally (i.e. symbionts with different roles may be found together more often).

The seven facultative symbionts found in the pea aphid are *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, *Rickettsiella viridis*, *Rickettsia*, *Spiroplasma*, and X-type. *H. defensa* is an anti-parasitoid symbiont (Oliver *et al*. 2003). *Serratia*

symbiotica may confer low levels of defense against parasitoids (Oliver *et al*. 2003), but is more commonly known to protect aphids against the negative fitness effects often caused by high temperatures (Russell & Moran 2006; Burke *et al*. 2010). *Rickettsiella* is an anti-pathogen symbiont (Łukasik *et al*. 2013). An additional advantage of *Rickettsiella* is its ability to change red aphids green. This color polymorphism will be beneficial depending on selective pressures from predation and parasitism. For example, ladybird beetles tend to eat red aphids and parasitoids seem to prefer to oviposit in green aphids (Losey *et al*. 1997). *R. insecticola* primarily protects against pathogenic fungi (Scarborough *et al*. 2005). *Rickettsia* and some strains of *Spiroplasma* are also primarily anti-pathogen symbionts and have been shown to defend against *Pandora neoaphidis* (Łukasik *et al*. 2013). Research suggests X-type might have potential defensive properties as it may improve *H. defensa* resistance to parasitoids especially under heat stress (Guay *et al*. 2009). X-type also appears to enhance thermotolerance and defense against *P. neoaphidis* when co-infecting with *Spiroplasma* (Heyworth & Ferrari, 2015).

The frequencies of these seven symbionts may be dictated by environmental factors. We have seen symbiont frequencies correlated with environmental changes in other systems. For example, climate conditions have been shown to dictate the presence of endophytes in *Lolium perenne* with plants in conditions with more drought being more likely to contain the endophytes (Gundel *et al*. 2011). Oliver *et al*. (2008) examined symbiont frequency fluctuation caused by selective pressures in the pea aphid system. In this lab study, conducted under heavily controlled conditions, they observed fluctuations in *H. defensa* frequencies in aphid populations exposed to the parasitoid *Aphidius ervi*. Their results showed an increase in *H. defensa* frequencies in the presence *A. ervi* and a

decrease in *H. defensa* frequencies in cages that did not contain *A. ervi*. Figure 1 from Oliver *et al*. (2008) shows the difference in *H. defensa* frequency between the aphids in the cage exposed to (a) *A. ervi* and (b) the cage absent of wasps.

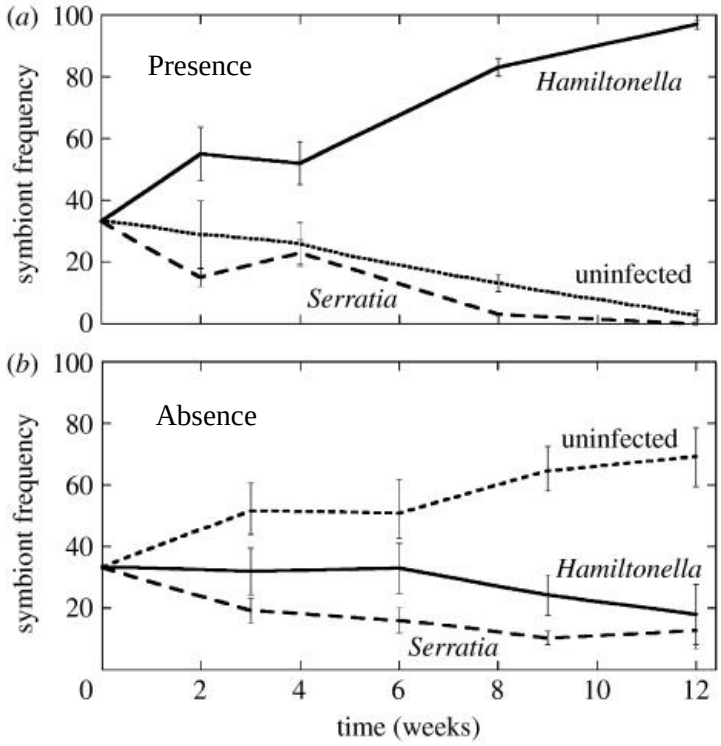


Figure 1. **Infection Frequencies of *A. pisum* Secondary Symbionts Over Time.** Aphids may be infected with either *Hamiltonella* (solid line), *Serratia* (long-dashed line) or uninfected with secondary symbionts (short-dashed line). (*a*) Infection frequencies (with s.e. at sampling points) in the presence of parasitic wasps and (*b*) infection frequencies in the absence of wasps (Oliver *et al*. 2008).

Smith *et al*. (2015) examined these trends more broadly in natural settings. They only saw the expected correlation between *H. defensa* frequencies driven by *A. ervi* frequencies in one of their three heavily sampled populations. They also found none of the predicted correlations between temperature and frequencies of anti-pathogenic

symbionts in the aphid populations sampled. This suggests that the roles symbionts play in aphid seasonal adaptation are still largely unknown, and also raises the possibility that currently known beneficial impacts of symbionts may not be the most relevant roles for these symbionts in natural conditions. It is likely that symbiont frequencies may not be closely correlated with environmental changes because there are other determinants of symbiont frequency in natural settings such as vertical transmission efficiency (Dykstra *et al*. 2014; Haselkorn & Jaenike, 2015), superinfection and/or hitchhiking (Smith *et al*. 2015).

The composition of the microbial communities living in pea aphids will be dictated in part by context-dependent benefits that individual symbionts provide, since these symbionts are living within the same organism and fighting for the same resources. These cohabitating symbionts may be able to exist within the aphid successful by either building alliances with each other and/or developing mechanisms to block other symbionts' defensive strategies. Microbial communities that pair together through vertical transmission over long time scales can develop the ability to collaborate and sabotage horizontally transmitted symbionts (Vautrin & Vavre, 2009). It is possible that symbionts could use these same techniques to prevent newly transmitted bacteria from colonizing in a particular aphid.

Smith (2015) studied populations of pea aphids from 6 states over 3 years and compared the expected frequencies of heritable facultative symbiont (HFS) pairings found using expected random binomial distributions to the actual observed frequencies of these pairings. These findings are summarized in Figure 2. Their results showed *Hamiltonella-Spiroplasma* was found less often than predicted in 3 host populations;

Hamiltonella-Rickettsia was found more often than predicted in 5 host populations; *Hamiltonella*-X-type was found more often than predicted in 9 host populations; *Serratia-Rickettsiella* was found more often than predicted in 13 host populations.

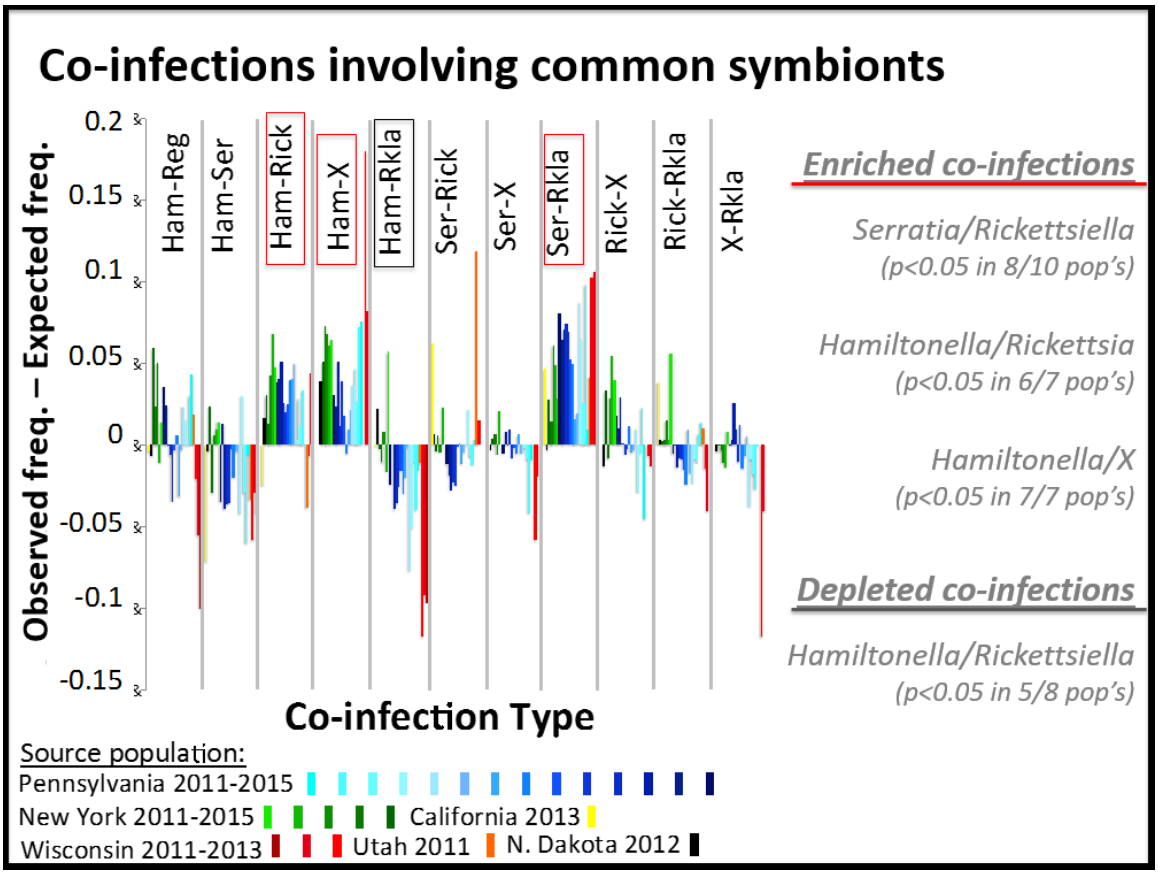


Figure 2: HFS Community Structuring in Pea Aphids. Differences in observed and expected frequencies in pairwise associations of HFS species in single aphid hosts.

Spiroplasma was found alone more often than predicted and negatively associated with several symbionts. These results are shown in Figure 3. Two of the most prevalent pairings, *Serratia-Rickettsiella* and *Hamiltonella-Rickettsia*, contain symbionts that can each confer some level of resistance to parasitoids and pathogens whereas *Hamiltonella*-X-type is a pairing that has been shown to improve anti-parasitoid protection under

extreme heat (Guay et al. 2009). Therefore, these pairings may have been selected for based on their ability to provide optimal seasonal adaptation for their aphid hosts. We are unsure of the mechanism, but it appears that common symbiont pairs may have gained an

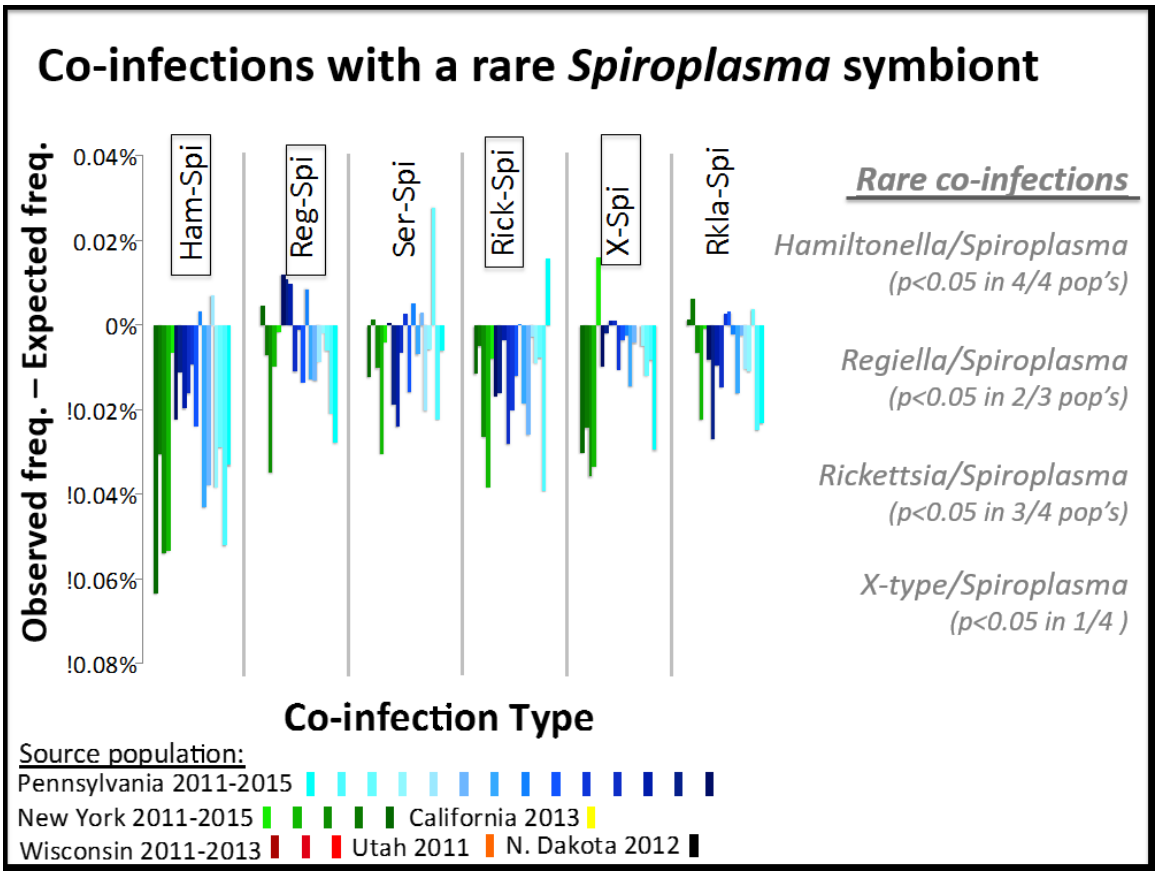


Figure 3: HFS Community Structuring in Pea Aphids. Differences in observed and expected frequencies in pairwise associations involving *Spiroplasma*.

advantage by building alliances and/or by developing resistance to each other's competitive strategies. By contrast, *Spiroplasma* seems to exist in aphids by using deleterious effects on other symbionts present in the aphids' microbiome.

The next step in examining these symbiont dynamics is quantitatively identifying the transmission efficiency of symbionts from one generation to the next. Aphids can acquire new symbionts via the horizontal transmission conduits listed above. However, little is known about the mechanisms involved in the spread and maintenance of these symbionts after they have been laterally acquired. The goal of this research is to determine the natural rate of maternal transmission for secondary symbionts in pea aphids while considering the role of transmission efficiency in the maintenance of symbionts and symbiont pairings and examining the factors that influence these transmission rates.

Materials and Methods

Field methods

Pea aphids (*Acyrthosiphon pisum*) were collected June-December 2013 and May-October 2014 in three separate alfalfa fields in Montgomery County, PA. In 2013, there were 8 collections all from one field, later named field I. In 2014, there were 18 different collections – 7 from field I, 6 from field II, and 5 from field III. Aphids were collected using beat sampling from plants separated by approximately 20 m to minimize re-sampling of the same clones. We targeted 4th instar aphids, which are just one developmental stage removed from adulthood. This helped to standardize the timing of reproduction by aphids in our experiments and, hence, the approximate birth order of F_1 aphids used for our screening.

Each F_0, or parental, aphid designated for our experiment was placed individually into a fine mesh sleeve, which was closed temporarily with a clothes pin. After all aphids had been prepared in this fashion, we selected a central location in each field for aphid placement. Individual shoots of alfalfa were manually brushed off and inspected to ensure elimination of other invertebrates. At this point, single shoots were enclosed within the mesh sleeves containing the individual 4^{th} instar aphids. Sleeve openings were twisted around the plant and secured with one clothes pin and a piece of tape. At the same time a single temperature probe was placed into a sleeve and secured in a mesh sleeve with a single alfalfa shoot in the exact same fashion, helping us to track temperature fluctuations. In a follow-up experiment to assess temperature differences inside and outside of these sleeves, we repeated this temperature probe caging for several probes, and placed them in proximity to probes held outside of sleeves.

Caged aphids were left in the field for 10 days (2013) or 8 days (2014). At the end of these periods, we recorded whether the F_0 parental aphid was still alive and the number of offspring produced. F_0 aphids were preserved in 95% ethanol at the time of collection and stored at -20°C prior to DNA extraction and symbiont screening. Their offspring (F_1 aphids) were placed onto fava bean leaves nourished through agar within petri dishes and grouped by stage of development with no more than 5 aphids on each dish—all siblings from a single mother. These aphids were then placed into an incubator and kept at 20 degrees C for 16 hours light and 8 hours dark until 10 days had passed since first reproduction. Due to previous documentation of secondary symbiont density increases into adulthood (Koga *et al*. 2003; Sakurai *et al*. 2005), we reasoned that this protocol would allow us to best address whether symbionts were present in these F_1

individuals. At this point all F_1's were placed into 95% ethanol and stored at -20°C prior to DNA extraction and symbiont screening (see below).

Laboratory methods

DNA extractions

DNA from preserved aphids was extracted following prior protocols (Russell et al. 2003). In 2014, we included blank extractions as two negative controls for every batch of 94 F_1, helping us to identify any contamination arising at the extraction stage. Prior to extraction, ethanol-preserved aphids were rinsed with a 6% bleach solution followed by distilled water. They were then flash-frozen in liquid nitrogen, crushed with a sterile plastic pestle and incubated at 65°C with lysis buffer (in 100 ml volume: 10 ml 8M Tris, 10 ml 0.5 M EDTA, 5 ml 2M NaCl, 20 ml Sucrose, 0.3g Sodium Dodecyl Sulfate) for 30 min. Following incubation, 8 M potassium acetate was added and samples were chilled for 40 minutes. Samples were then centrifuged and supernatant discarded prior to washing the pellet with 95% ethanol, ice-cold 70% ethanol, and finally 100% ethanol. Samples were dried under vacuum and suspended in 60 µl low TE (in 100 ml volume: 5 ml 8M Tris, 1 ml 0.5 M EDTA) prior to long term storage at -20 ◦C. DNA template quality was verified for all extractions included in analyses using a Polymerase Chain Reaction (PCR) assay to detect *Buchnera aphidicola*, the obligate primary symbiont harbored by all pea aphids (Table S1).

In total we generated 123 quality DNA extractions from F_0 aphids and 833 from their F_1. Although we extracted DNA from an additional 106 offspring, these were from

F_0 mothers without quality DNA extractions. Therefore, we did not use these F_1 samples to measure vertical transmission due to the uncertain infection status of their mothers.

PCR and DNA Sequence Confirmation

To test individual aphids for the seven species of facultative symbionts found in United States populations, DNA samples were subjected to diagnostic PCRs for each symbiont to amplify a fragment of the 16S rRNA gene. Primer sequences and thermocycling conditions used for diagnostic PCRs are listed in Table S1. All PCR amplifications for symbiont screening were performed using 10 µL volumes including: 5 µl of the reaction mix MyTaqTM red mix (Bioline Reagents Ltd., London, UK), 1 µl of each the forward and reverse primers, 2.4 µl of ddH2O and 0.6 µl DNA. Symbionts were scored as present if a band existed on the gel and coincided with the band length of the positive control. If a band was present for the negative control the entire reaction was considered contaminated and the PCR was rerun.

To determine whether faint bands amplifying from our 2013 samples were truly indicative of symbiont presence, we sent out such ambiguous samples for Sanger sequencing. Using the above PCR protocols samples were first re-amplified in a 20 µl reaction volume, using the same diagnostic primers. We purified these samples using *E. coli* Exonuclease I and Antarctic Phosphatase (New England BioLabs, Inc., Ipswich, MA). Samples were then shipped to Eurofins MWG Operon (Huntsville, AL), where sequencing took place using one of the PCR primers. After manual editing in Codon Code Aligner v.4.0.3 (Centerville, MA) aligned sequences of each symbiont 16S rRNA genes were used in BLASTn searches against NCBI's nucleotide database. Out of 108

samples assessed in this manner, 101 were confirmed to be positive for the targeted symbiont. A further 7 generated poor quality sequence. We, hence, reasoned that faint bands were a good indicator of symbiont presence. For this reason, we shifted our method for re-assessing ambiguous (i.e. faint band) samples for our 2014 collections, moving to a more affordable method of re-amplifying such samples with 10 additional PCR cycles.

For re-screening of faint bands, PCR amplifications were performed using 15 µL volumes including: 7.5 µl of the reaction mix MyTaqTM red mix (Bioline Reagents Ltd., London, UK), 1.5 µl of the forward and reverse primer, 3.6 µl of ddH2O and 0.9 µl DNA. The product was run in the thermocycler as specified in Table S1 with the 10 additional cycles to aid in amplification of potentially low density infection.

In our PCR screening we found six of the 123 examined aphid lines were potentially impacted by contamination at the extraction stage. In four of these lines, the F_0 aphids were processed in an extraction batch where there was a faint band present for blank extractions (one or more) in the *Rickettisella* PCR (line IDs: 30_03, 5 F_1's; 30_10, 7 F_1's; 30_20, 5 F_1's; 31_05, 9 F_1's). In the fifth line, there were faint *Rickettisella* bands for the blank extractions generated in the same extraction batch with most of the F_1 aphids from the line in question (16_13, 6 F_1's). In the sixth line, two of the seven F_1 aphids were part of an extraction batch that contained blank DNA extractions yielding a faint positive *Serratia* band (30_21, 7 F_1's). Potential contamination did not create ambiguity in determining transmission efficiency for any of the other symbionts or lines. Although PCR products for the secondary symbionts in the potentially affected specimens were generally strong, we performed our statistical analyses both with and without these six ambiguous lines to protect against the possibility of spurious results.

Microsatellite genotyping

To identify potential re-sampling of the same host clones used in our transmission experiments, microsatellite genotyping was performed on all F_0 aphids. A subset of F_1 aphids were also genotyped to ensure they were indeed the offspring of F_0 aphids from the same mesh sleeves. Specifically, F_1 aphids were genotyped if the symbionts they harbored differed from those harbored by their putative F_0 mother. For each symbiont community type in a F_1 cohort with such disagreement we genotyped at least one representative aphid. When F_0 and F_1 microsatellite genotypes matched, this allowed us to include such aphids in our statistical analyses on vertical transmission. Of the 230 F_1 aphid symbiont profiles checked, there were 10 F_1 aphids with aberrant symbiont profiles whose microsatellite genotypes diverged from that of their putative mothers. In six of these cases, only the aphids that did not match the F_0 parental aphid from the same mesh sleeve were removed from our analyses. In the remaining four cases, the whole line was removed as these cohorts had small F_1 numbers, and therefore, the majority of the line was compromised and it had to be excluded. Due to the low rate of contamination in our study, three F_1 aphids with ambiguous genotyping results (i.e. no clear alleles due to PCR product yielding low concentrations) at one or more loci were kept in our analyses and only used in the "with ambiguous cases" statistical runs.

For genotyping, we characterized aphids at five microsatellite loci: S23, S24, ApH10M, APF08M, and S30 (Caillaud *et al.* 2004; Wilson *et al.* 2004). Each locus was amplified in a multiplex reaction containing all primer pairs, with the forward primers labeled with the fluorescent dyes 6-FAM, VIC, 6-FAM, NED, and PET, respectively. Amplification was performed in 10 µl reactions containing 6.25 µl My*Taq* Red Mix,

forward and reverse primers at varying volumes (i.e. 0.1 µl for S23, S24, Aph10M; 0.2 µl for ApF08M, and 0.3 µl for S30), 0.4 µl $MgCl_2$ @ 50 mM, 0.65 µl H_20 and 0.5 µl DNA. Thermocycling conditions used were from Wilson *et al.* (2004), PMS1: 94 °C for 2 mins, followed by one cycle of 62 °C for 30 sec, 72 °C for 45 sec, and 94 °C for 15 sec; one cycle of 61°C for 30 sec, 72 °C for 45 sec, and 94 °C for 15 sec; one cycle of 59 °C for 30 sec, 72 °C for 45 sec, and 94 °C for 15 sec; one cycle of 57 °C for 30 sec, 72 °C for 45 sec, 94 °C for 15 sec; 30 cycles of 55 °C for 30 sec, 72 °C for 45 sec, and 94 °C for 15 sec; and, finally, one cycle of 72 °C for 2 mins. After the presence of products within the expected size range was confirmed by gel electrophoresis, products were diluted three-fold and submitted for fragment sizing on an Applied Biosystems 3130XL at the University of Pennsylvania Sequencing Center. Chromatograms were analyzed using GeneMarker V2.2.0.

Statistical Analysis

All analyses were carried out using the lme4 package in R version 3.3.2. The transmission efficiency of *Rickettsiella*, *Serratia*, *Hamiltonella*, and *Spiroplasma* was determined using a repeated measures generalized linear model. The binominal dependent variable in each model was the presence or absence of each symbiont. This analysis treats each aphid as a separate replicate. Each model was run both with the ambiguous cases included and without the ambiguous cases. To determine differences in transmission efficiency between groups of infection types, the model used for

Rickettsiella included the infection status of the F_0 and the average temperature as fixed effects and the F_0 cohort and the genotype group as random or block effects.

When we observed the variance in transmission associated with the random effects, we found it to be low, which means that the F_0 aphid ID and the clone ID probably do not have a large effect on the probability of transmission. One thing to note here is that the variance of the clone ID is much larger than the variance of the F_0 aphid ID, which means its effect is much more important. The random effects for each F_0 were also inspected manually to see if there are any with a particularly large value. The CloneID's with the largest random effects in analysis with the ambiguous cases included were MS_24 (-4.17) and MS_50 (-4.00). In the analysis without the ambiguous cases the CloneID's with the largest random effects were MS_25 (-2.61) and MS_62 (-2.71).

For the analysis of *Serratia* loss, we could not use the complete model used for the *Rickettsiella* analysis. Average temperature had to be removed from the model because the AIC values in the drop one function were not 2 units apart, which indicates that average temperature should not be part of the statistical model in this case. The model used for *Serratia* included infection status of the F_0 as a fixed effect and the F_0 cohort and the genotype group as random or block effects. The model had to be simplified further for the *Hamiltonella* and the *Spiroplasma* data due to small sample sizes. For these two analyses we only used one fixed effect, the F_0 infection status.

To look at the differences in transmission for each symbiont, we performed a Tukey's post hoc test. We found statistically significant differences between several of the groups used in the comparisons for *Rickettsiella*, *Serratia* and *Hamiltonella*. There

were no statistically significant differences between the bins used for *Spiroplasma*. These results are summarized in Table 3.

Results

When we first analyzed our results, we looked like transmission efficiency for each individual symbiont. Figure 4 summarizes the transmission efficiency from F_0 mothers to F_1 offspring for each individual symbiont. These results contrast with the nearly perfect transmission efficiency seen in laboratory studies. The greatest transmission failure appears to occur for *Hamiltonella* where we only see *Hamiltonella* transferred to 75% of F_1's whose mothers' had *Hamiltonella*. *Serratia* and *Spiroplasma* had the next lowest rates of transmission, 87% and 88%, respectively. The transmission rate for *Rickettsiella* was 90% and X-type was 93%. *Rickettsia* and *Regiella* had perfect rates of transmission from the very small numbers of infected F_0 in our study.

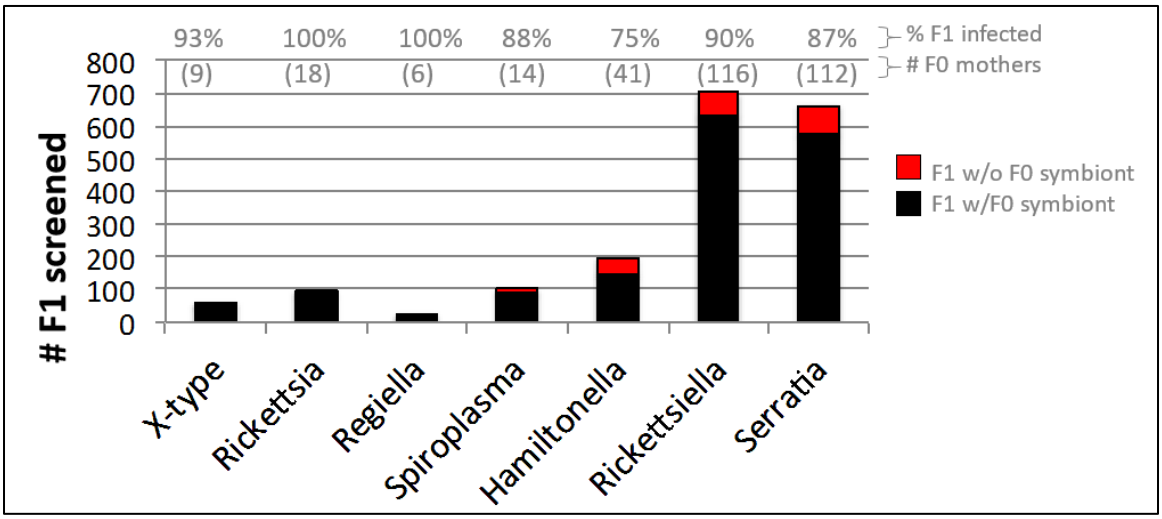


Figure 4. Individual Symbiont Loss

We were also interested in how infection types affected transmission efficiency. Using the statistical models described in the methods section, we aimed to determine whether the infection status of pea aphids shapes the rate of vertical transmission of individual symbionts from asexual aphids to their offspring. Due to the large number of symbiont communities observed and our expectations that co-infection would shape transmission rates, we binned symbionts into separate "treatment" categories based on whether they included a common symbiont pairing (or trio), whether symbionts were found alone, whether common pairings co-infected with one or more other symbionts, and whether *Spiroplasma* symbionts co-infected with the focal symbiont (across any co-infection context; see below for rationale).

For separate analyses on the transmission of *Rickettsiella* and *Serratia*, F_0 mothers were binned into five categories. To define these groupings we started with the observation that *Serratia* commonly co-infects aphids with *Rickettsiella*. We were also interested in whether the presence of a third or fourth symbiont co-infecting with *Rickettsiella* and *Serratia* might impact transmission, lumping community types into separate treatment groups based on whether there was just one or multiple additional co-infecting species. Since *Spiroplasma* lives with other symbionts less often than expected, we included a separate treatment category in our model that lumped community types including this symbiont along-side the common partners of *Rickettsiella* and *Serratia*.

Table 1 lists the abbreviations used throughout the analysis and Table 2 lists the bins for each symbiont along with the total F_0 and F_1 counts for each bin. The analysis was done both with the ambiguous cases included and without the ambiguous cases. Ambiguous cases include instances where there may have been questions about the true

Table 1. **Symbiont Abbreviations Used for Statistical Analysis**

Rickettsiella	Rkla
Serratia symbiotica	Ser
Hamiltonella defensa	Ham
Rickettsia	Rick
Regiella insecticola	Reg
X-*type*	X-type
Spiroplasma	Spiro

infection status of an F_0 or an F_1 because of possible contamination, slight differences in microsatellite genotyping results, and inconclusive sequencing results. In Table 2, the totals for each group with the ambiguous cases excluded are listed in parentheses.

The F_0 mothers infected with *Rickettsiella* were binned into five categories. Rkla.Ser contains aphid lines established by F_0 aphids that were infected by *Rickettsiella* and *Serratia* only. The second group, Rkla.Ser+1, contains lines that were infected with *Rickettsiella*, *Serratia* and one other facultative symbiont except *Spiroplasma*. The third group, Rkla.Ser+>=2, contains lines that were infected with *Rickettsiella*, *Serratia,* and two or more other symbionts, but does not include groups with *Spiroplasma.* The fourth group, Rkla.Ser.Spiro, contains lines that were infected with *Rickettsiella*, *Serratia*, and *Spiroplasma*. The fifth group, Rklaw/oSer, contains lines that are infected with *Rickettsiella*, but not *Serratia*. This group included both the *Rickettsiella* single infection along with *Rickettisella* paired with other symbionts in a double or triple infection. The *Serratia* bins were organized in the same fashion as the *Rickettsiella* bins.

For *H. defensa*, F_0 mothers were binned into five categories. To define these groupings we started with the observation that *H. defensa* commonly co-infects aphids

with *Rickettsia* and *X-type*. Since *Spiroplasma* lives with other symbionts less often than expected, we decided not to bin the Ham.Rkla.Ser.Spiro group in with the other Ham.Other subgroups and instead included a separate treatment category for the aphids with the Ham.Rkla.Ser.Spiro infection status. The title of this group was shortened to Ham.Spiro.

The first group, Ham.Rick, contains aphid lines established by F_0 aphids that were infected by *H. defensa* and *Rickettsia*. The second group, Ham.X, contains lines that were infected by *H. defensa* and X-type. The third group, Ham.Rick.X, contains lines that were infected with *H. defensa*, *Rickettsia,* and X-type. The fourth group, Ham.Spiro, contains lines that were infected with *H. defensa* and *Spiroplasma*. The fifth group, Ham.Other, contains lines that are infected with *H. defensa*, but not *Rickettsia* or *X-type.* Ideally, we would have analyzed the presence of *Rickettsia* and *X-type's* effect on the transmission of *H. defensa* in two separate analyses. However, we did not have sample sizes large enough to run these analyses.

For *Spiroplasma*, F_0 mothers were binned into three categories. The first group, Spiro alone, contains aphid lines established by F_0 aphids that were infected by *Spiroplasma* only. The second group, Spiro+1, contains lines that were infected by *Spiroplasma* and one other symbiont. The third group, Spiro+>=2, contains lines that were infected with *Spiroplasma* and two or more other symbionts.

Table 2. Symbiont Groups Used for Statistical Analysis. Bins for each symbiont along with the total F_0 and F_1 counts for each bin. The totals with the ambiguous cases removed are listed in parenthesis.

***Rickettsiella* Bins**				
Rkla.Ser **F_0: 71 (57)** **F_1: 442 (376)**	**Rkla.Ser+1** **F_0: 17 (14)** **F_1: 88 (69)**	**Rkla.Ser+>=2** **F_0: 6 (4)** **F_1: 33 (17)**	**Rkla.Ser.Spiro** **F_0: 6 (5)** **F_1:37 (32)**	**Rklaw/oSer** **F_0: 17 (11)** **F_1: 98 (64)**
Rkla.Ser	Rkla.Ser.Ham Rkla.Ser.Reg Rkla.Ser.Rick	Rkla.Ser.Ham.Reg Rkla.Ser.Ham.Rick Rkla.Ser.Ham.Xtype	Rkla.Ser.Spiro Rkla.Ser.Ham.Spiro	Rkla Rkla.Ham Rkla.Ham.Reg Rkla.Ham.Rick Rkla.Ham.Xtype Rkla.Spiro
***Serratia* Bins**				
Ser.Rkla **F_0: 71 (57)** **F_1: 442 (376)**	**Ser.Rkla+1** **F_0: 17 (14)** **F_1: 88 (69)**	**Ser.Rkla+>=2** **F_0: 6 (4)** **F_1: 33 (17)**	**Ser.Rkla.Spiro** **F_0: 6 (5)** **F_1: 37 (32)**	**Serw/oRkla** **F_0: 14 (9)** **F_1: 60 (47)**
Ser.Rkla	Ser.Rkla.Ham Ser.Rkla.Reg Ser.Rkla.Rick	Ser.Rkla.Ham.Reg Ser.Rkla.Ham.Rick Ser.Rkla.Ham.Xtype	Ser.Rkla.Ham.Spiro Ser.Rkla.Spiro	Ser Ser.Ham Ser.Ham.Reg Ser.Ham.Rick.Xtype Ser.Ham.Xtype Ser.Spiro
***Hamiltonella* Bins**				
Ham.Rick **F_0: 10 (7)** **F_1: 37 (24)**	**Ham.X** **F_0: 6 (4)** **F_1: 31 (12)**	**HamRick.X** **F_0: 3 (3)** **F_1: 22 (19)**	**Ham.Spiro** **F_0: 2 (1)** **F_1: 19 (14)**	**Ham.Other** **F_0: 21 (14)** **F_1:83 (61)**
Ham.Rick.Rkla Ham.Rick.Ser Ham.Rick.Rkla.Ser	Ham.Rkla.Ser.Xtype Ham.Rkla.Xtype Ham.Ser.Xtype	Ham.Rick.Ser.Xtype	Ham.Rkla.Ser.Spiro	Ham Ham.reg Ham.Rkla Ham.Rkla.Reg Ham.Rkla.Ser Ham.Rkla.Ser.Reg Ham.Ser.Reg Ham.Ser
***Spiroplasma* Bins**				
Spiro alone **F_0: 4 (4)** **F_1: 36 (29)**	**Spiro+1** **F_0: 4 (3)** **F_1: 25 (17)**	**Spiro+>=2** **F_0: 6 (5)** **F_1: 37 (32)**		
Spiro	Spiro.Rkla Spiro.Ser	Spiro.Rkla.Ser.Ham Spiro.Rkla.Ser		

Figure 5 is a graphical representation of the transmission loss in the symbiont groups used for the statistical analysis of *Rickettsiella* and *Serratia* loss. Here we can see that when *Rickettsiella* and *Serratia* are paired together alone, this appears to result in the highest transmission success for both *Rickettsiella* and *Serratia*. The addition of one additional symbiont to the *Rickettsiella.Serratia* pairing does not appear to have much of a negative impact on transmission efficiency. However, the addition of two or more symbionts does seem to start to negatively impact transmission success. We see the lowest rates of transmission when *Spiroplasma* is present. There are also relatively low levels of transmission for *Rickettsiella* when *Serratia* is not present and low levels of transmission for *Serratia* when *Rickettsiella* is not present.

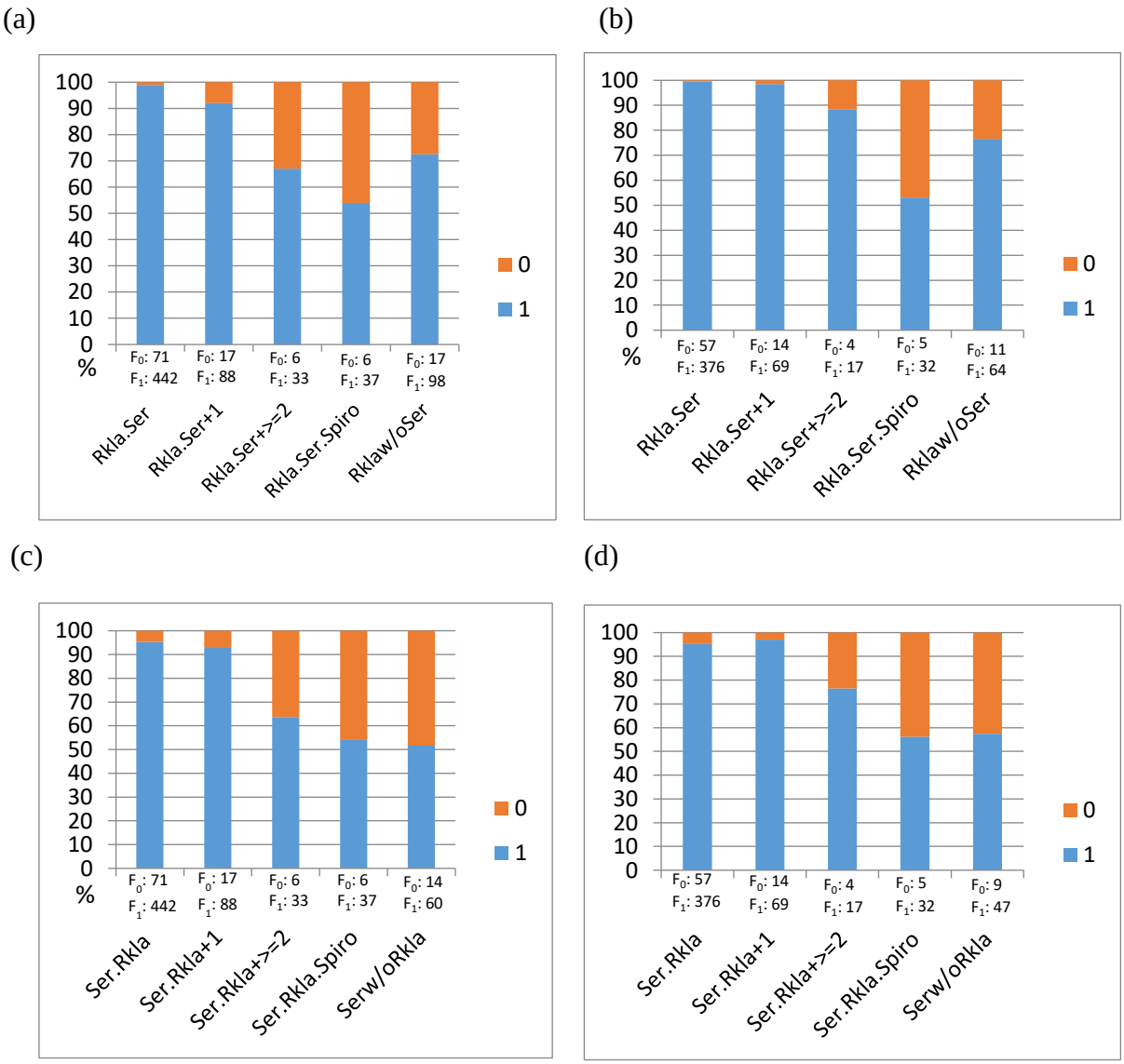


Figure 5. ***Rickettsiella* and *Serratia* Loss** (a) *Rickettsiella* loss with ambiguous cases included (b) *Rickettsiella* loss without ambiguous cases (c) *Serratia* loss with ambiguous cases included (d) *Serratia* loss without ambiguous cases. The vertical axis shows the percentage of successful vertical transmission. The blue sections on the bar graph labeled "1" indicate the percentage of F_1's with symbiont presence and the orange sections labeled "0" indicate the percentage of F_1's with symbiont absence. F_0 and F_1 counts for each bin are listed below each bar.

Figure 6 is a graphical representation of the transmission loss in the symbiont groups used for the statistical analysis of *Hamiltonella* loss. The highest rates of

transmission occur when *Hamiltonella* is paired with both *Rickettsia* and X-type. However, it is important to note here that there were only three clonal lines with the infection status Ham.Rick.X so these results could be skewed by small sample size biases. We also see what looks like extremely high levels of *Hamiltonella* loss when *Spiroplasma* is present. It is important to note, though, that there are only two clonal lines that contain *Spiroplasma*. These lines also harbored both *Rickettsiella* and *Serratia*. Therefore, it is unclear how influential the presence of *Spiroplasma* is on *Hamiltonella* transmission.

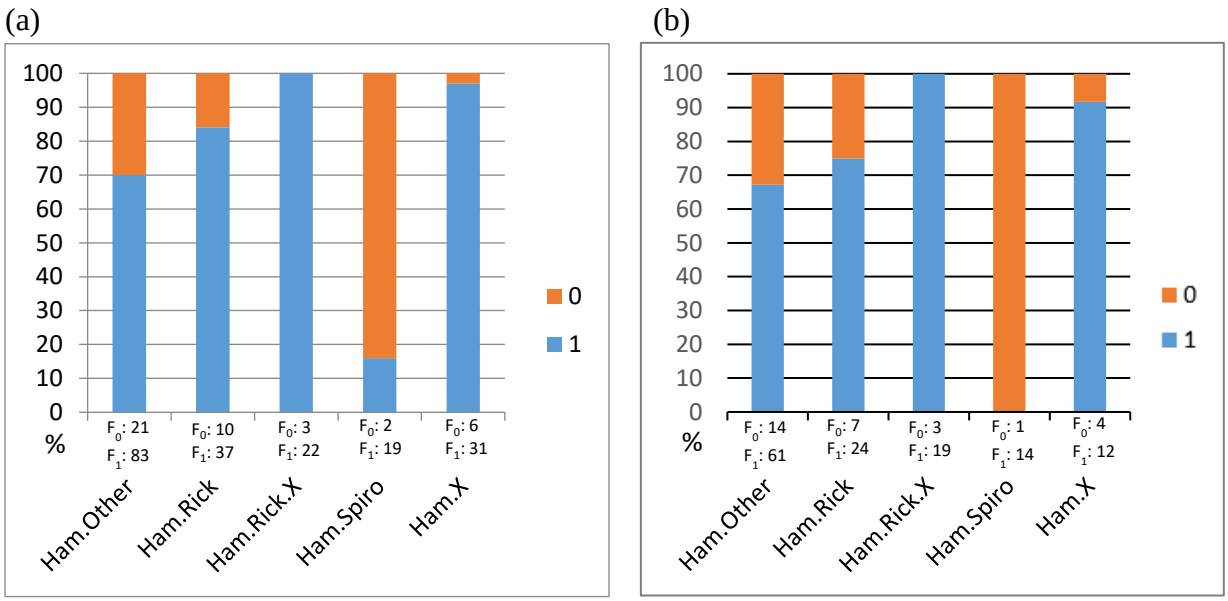


Figure 6. ***Hamiltonella*** **Loss** (a) *Hamiltonella* loss with ambiguous cases (b) *Hamiltonella* loss without ambiguous cases. The vertical axis shows the percentage of successful vertical transmission. The blue sections on the bar graph labeled "1" indicate the percentage of F_1's with symbiont presence and the orange sections labeled "0" indicate the percentage of F_1's with symbiont absence. F_0 and F_1 counts for each bin are listed below each bar.

We were able to obtain larger sample sizes for the Ham.Rick, Ham.X, and Ham.Other groups. Within these three groups, it looks like the highest rates of transmission occurred when *Hamiltonella* was paired with X-type. The next highest was when *Hamiltonella* paired with *Rickettsia*. The rates appear to drop when *Hamiltonella* is along or pair with something other than *Rickettisa* or X-type.

Figure 7 shows the transmission loss for *Spiroplasma*. All three groups had relatively low sample sizes and there were no significant differences between them. Overall, *Spiroplasma* was transmitted at high rates with no clear strong effect of co-infection.

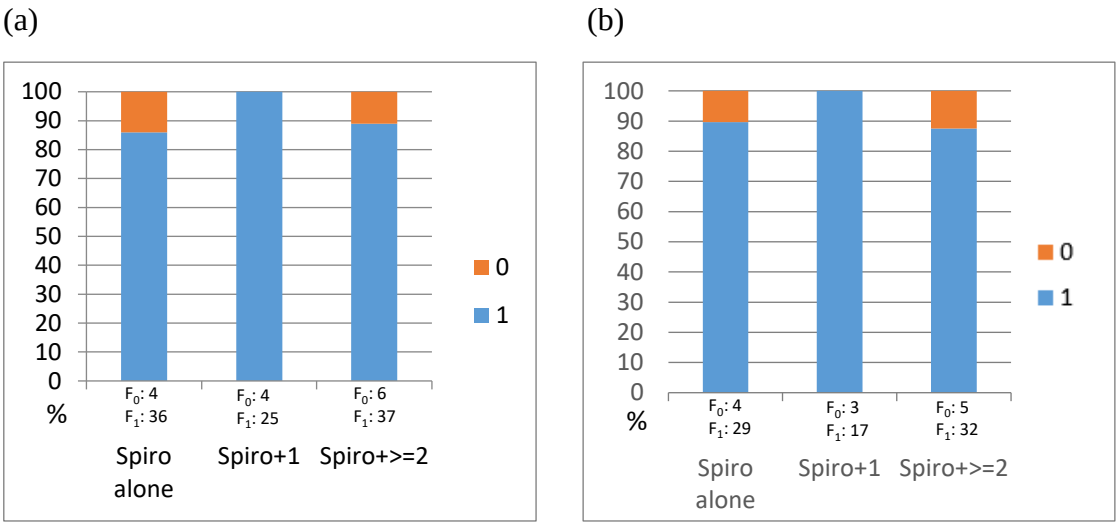


Figure 7. *Spiroplasma* Loss (a) *Spiroplasma* loss with ambiguous cases (b) *Spiroplasma* loss without ambiguous cases. The vertical axis shows the percentage of successful vertical transmission. The blue sections on the bar graph labeled "1" indicate the percentage of F_1's with symbiont presence and the orange sections labeled "0" indicate the percentage of F_1's with symbiont absence. F_0 and F_1 counts for each bin are listed below each bar.

The statistically significant results found in our dataset are listed in Table 3.

Table 3. Statistically Significant Symbiont Group Comparisons

Symbiont Group Comparisons	With Ambiguous Cases (p-value)	Without Ambiguous Cases (p-value)
Rickettsiella		
Rkla.Ser vs. Rklaw/o Ser	**<0.001**	**<0.001**
Rkla.Ser vs. Rkla.Ser.Spiro	**0.0271**	**0.0108**
Rklaw/oSer vs. Rkla.Ser+1	**0.0466**	0.3559
Rkla.Ser.Spiro vs. Rkla.Ser+1	0.0816	0.2055
Rkla.Ser vs. Rkla.Ser+>=2	0.1046	0.5554
Rkla.Ser+1 vs. Rkla.Ser+>=2	0.1673	0.8294
Rkla.Ser.Spiro vs. Rkla.Ser+>=2	0.9912	0.8227
Rklaw/oSer vs. Rkla.Ser.Spiro	0.9935	0.9662
Rklaw/oSer vs. Rkla.Ser+>=2	0.9999	0.9648
Rkla.Ser vs. Rkla.Ser+1	1.0000	0.9971
Serratia		
Ser.Rkla vs. Serw/oRkla	**<0.001**	**0.0065**
Serw/oRkla vs. Ser.Rkla+1	**0.0235**	**0.0237**
Ser.Rkla+>=2 vs. Ser.Rkla	0.1022	0.8347
Ser.Rkla vs. Ser.Rkla.Spiro	0.1167	0.3665
Ser.Rkla+1 vs. Ser.Rkla+>=2	0.2771	0.7339
Ser.Rkla+1 vs. Ser.Rkla.Spiro	0.3022	0.3299
Serw/oRkla vs. Ser.Rkla.Spiro	0.9952	0.9562
Ser.Rkla vs. Ser.Rkla+1	0.9967	0.9894
Serw/oRkla vs. Ser.Rkla+>=2	0.9992	0.8938
Ser.Rkla.Spiro vs. Ser.Rkla+>=2	1.0000	0.9966
Hamiltonella		
Ham.Rick vs. Ham.Spiro	**0.00012**	1.000
Ham.X vs. Ham.Spiro	**0.00015**	1.000
Ham.Other vs. Ham.Spiro	**0.00117**	1.000
Ham.Other vs. Ham.X	0.07482	0.440
Ham.Rick vs. Ham.X	0.43832	0.727
Ham.Other vs. Ham.Rick	0.43999	0.939
Ham.Rick.X vs. Ham.Other	1.00000	1.000
Ham.Rick.X vs. Ham.Rick	1.00000	1.000
Ham.Rick.X vs. Ham.Spiro	1.00000	1.000
Ham.Rick.X vs. Ham.X	1.00000	1.000

We found a statistically significant difference between Rkla.Ser and Rklaw/oSer. This indicates that the presence of *Serratia* does increase the transmission efficiency of

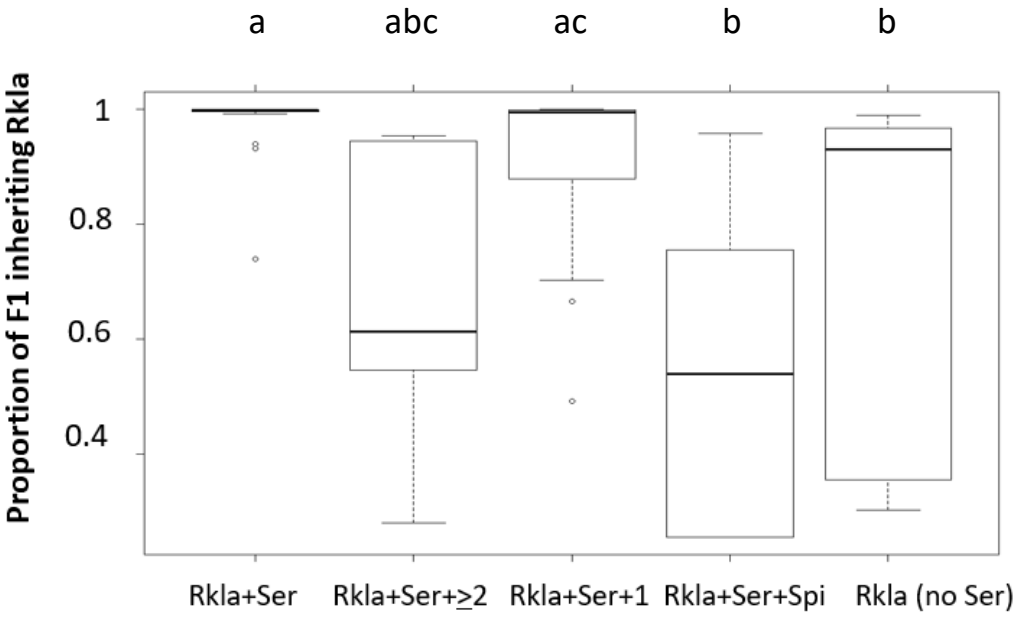
Rickettsiella. There was also a statistically significant difference between Rkla.Ser and Rkla.Ser.Spiro, which means *Spiroplasma* reduces the transmission efficiency of *Rickettsiella*. The difference between Rklaw/oSer and Rkla.Ser+1 was only a borderline significance that was no longer significant when we removed the ambiguous cases. Thus, at this point we cannot make any assumptions about the comparison between these two groups.

For *Serratia*, we again see a difference between Ser.Rkla and Serw/oRkla that is statistically significant. Therefore, we know that *Rickettsiella* has a positive impact on *Serratia* transmission. There is also a statistically significant difference between Serw/oRkla and Ser.Rkla+1. The difference between Ser.Rkla and Ser.Rkla.Spiro is not statistically significant. Therefore, we do not have enough evidence to say *Spiroplasma* decreases *Serratia* transmission efficiency.

In the *Hamiltonella* comparisons, the Ham.Spiro group was different from Ham.Rick, Ham.X, and Ham.Other. Therefore, it appears that the transmission efficiency of *Hamiltonella* is negatively impacted by the presence of Spiroplasma. Again, it is important to note here that the Ham.Spiro group was comprised of two clonal lines that each also harbored *Rickettsiella* and *Serratia* in addition to *Spiroplasma*.

These comparisons were also examined using the compact letter display of the general linear model hypothesis test. Figures 8, 9, and 10 show the proportion of F_1 inheriting the *Rickettsiella*, *Serratia*, and *Hamiltonella*, respectively, for each group. The hat categories displayed on the top of each infection type indicate the statistical significance. Hat categories without any shared letters show significant differences, where as those sharing letters show no differences.

(a)



(b)

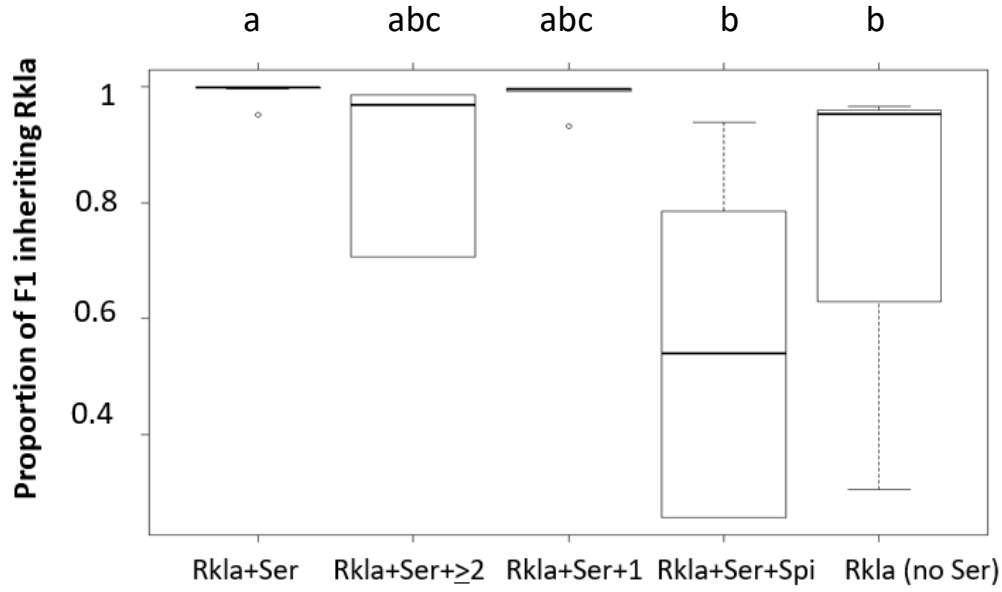
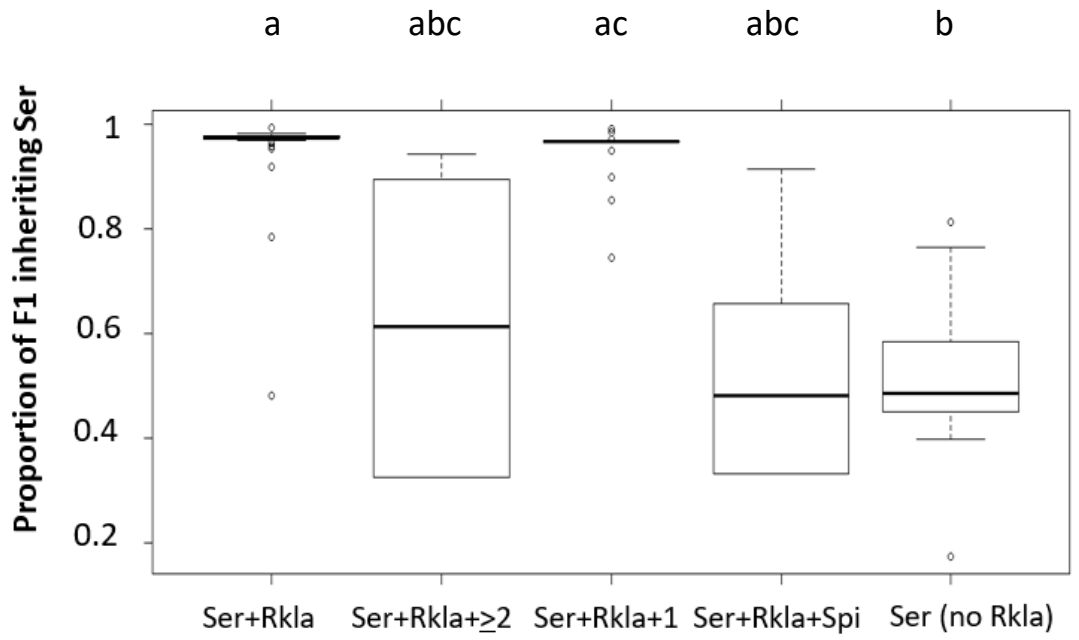


Figure 8. Compact Letter Displays – *Rickettsiella* (a) *Rickettsiella* with ambiguous cases (b) *Rickettsiella* without ambiguous cases

(a)



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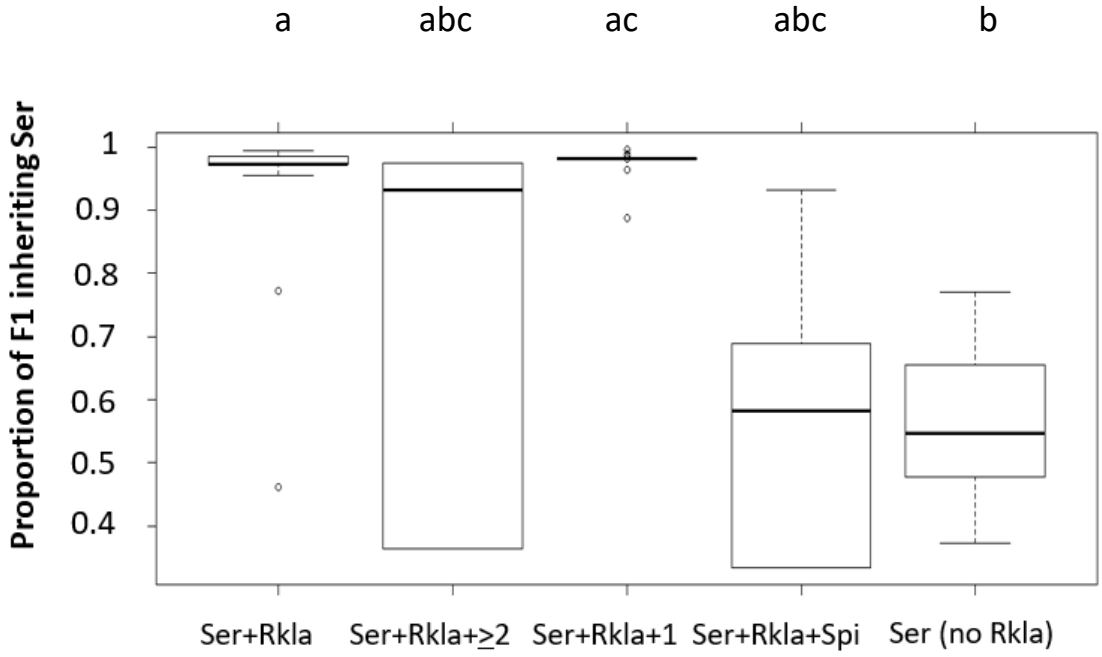
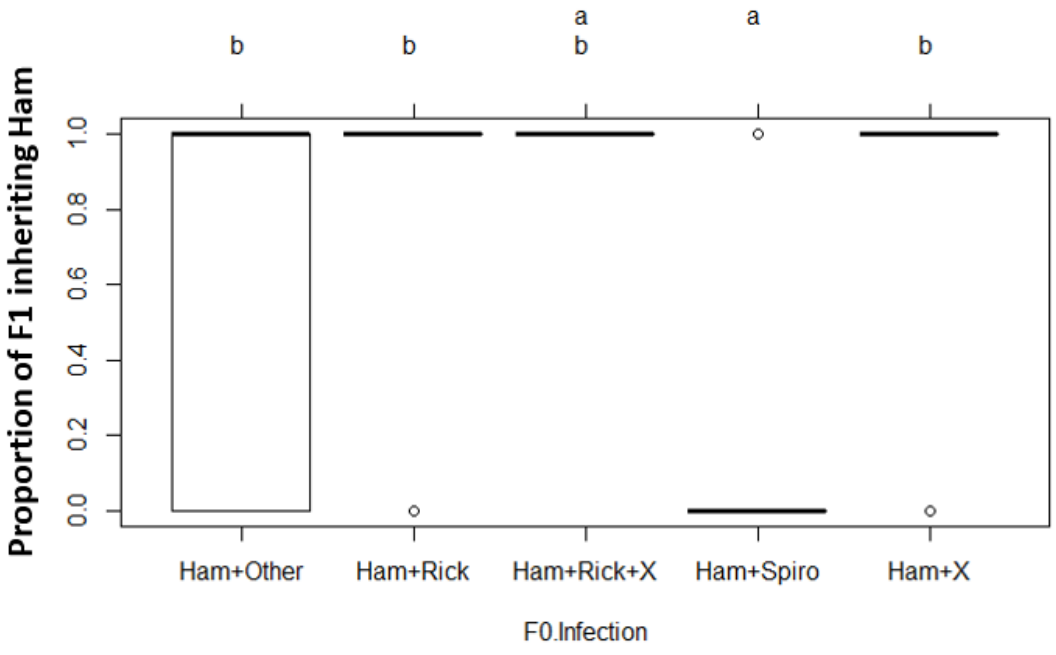


Figure 9. Compact Letter Displays –*Serratia* (a) *Serratia* with ambiguous cases (b) *Serratia* without ambiguous cases

(a)



(b)

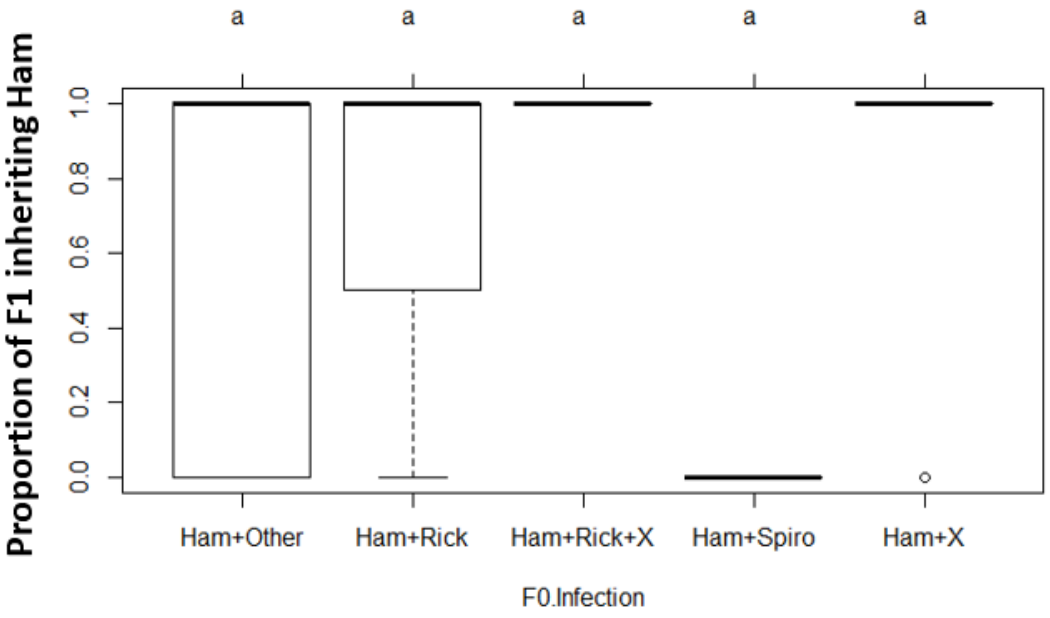


Figure 10. **Compact Letter Displays – *Hamiltonella*** (a) *Hamiltonella* with ambiguous cases (b) *Hamiltonella* without ambiguous cases

Table 4 summarizes the predicted probability of transmission loss for each group of F_0 infection types. These results were compiled for each group by predicting the probability of transmission failure for each F_1, grouping the F_0 aphids by the aforementioned F_0 infection categories, and then finding the average of these probabilities. Table 4 shows both the average probability for each infection group and the standard deviation for that probability.

Table 4. Predicted Probabilities of Transmission Efficiency

	With Ambiguous Cases		Without Ambiguous cases	
	Average of Probability	StdDev of Probability	Average of Probability	StdDev of Probability
Rickettsiella				
Rkla.Ser alone	0.9908	0.0326	0.9961	0.0103
Rkla.Ser+1	0.9244	0.1243	0.9910	0.0168
Rklaw/oSer	0.7295	0.2845	0.7694	0.2662
Rkla.Ser+>=2	0.6742	0.2457	0.8965	0.1265
Rkla.Ser.Spiro	0.5465	0.2683	0.5379	0.2833
Serratia				
Ser.Rkla alone	0.9548	0.0865	0.9541	0.0899
Ser.Rkla+1	0.9383	0.0718	0.9773	0.0234
Ser.Rkla+>=2	0.6410	0.2455	0.7760	0.2749
Ser.Rkla.Spiro	0.5435	0.2169	0.5670	0.2404
Serw/oRkla	0.5188	0.1529	0.5769	0.1074
Hamiltonella				
Ham.Other	0.6987952	2.63289E-08	0.6721311	0
Ham.Rick	0.8378378	2.62832E-08	0.75	0
Ham.Rick.X	1	0	1	0
Ham.Spiro	0.1578947	3.9268E-09	8.64687E-09	1.23168E-16
Ham.X	0.9677419	0	0.9166667	0
Spiroplasma				
Spiro	0.8611111	2.015E-08	0.8965517	0
Spiro+>=2	0.8918919	2.22133E-08	0.875	0
Spiro+1	1	0	1	0

We can see the highest predicted probability of transmission for *Rickettsiella* occurs when it is paired alone with *Serratia*. The addition of one other symbiont does not lower this probability by a large margin. However, when there are two or more symbionts added, we see a big drop in the predicted probability of transmission. When *Spiroplasma* is added, the predicted probability reduces to about 55%. We see similar trends for *Serratia*. However, for *Serratia* the largest drop in transmission efficiency happens when *Serratia* is found without *Rickettsiella*.

For Hamiltonella, the predicted probability of transmission is highest for Ham.Rick.X followed by Ham.x and Ham.Rick. The probability of transmission for Ham.Spiro is very low. For Spiroplasma, all of the predicted probabilities of transmission are relatively high.

Discussion

Despite potential benefits accrued by hosts harboring facultative symbionts, it is rare for facultative symbionts to reach fixation in field populations. Many facultative symbionts rely on successful maternal transfer to remain at high frequencies within a host population; and even the most beneficial symbionts can remain at intermediate frequencies due to modest rates of transmission failure. For example, a protective *Spiroplasma* symbiont of *Drosophila neotestacea* (with no cost in the absence of the nematode parasite targeted by this symbiont) is found in close to 80% of *Drosophila neotestacea* individuals in eastern North America. Jaenike *et al.* (2010) were able to accurately predict this infection prevalence as being close to the predicted equilibrium

frequency obtained by plugging estimated costs, benefits, and transmission rates (observed in the lab) into the equilibrium equation $\hat{P} = 1 - \left(\frac{1-\beta}{s} \right)$. In this equation, $\hat{P}$ is expected equilibrium prevalence, s is selective advantage of infected over uninfected cytoplasmic lineages and β is fidelity of maternal transmission of the symbiont. Here they estimated transmission efficiency to be 97% (or β=0.97) and obtained equilibrium results that matched the frequency of *Spiroplasma* in natural populations. The results of Jaenike *et al*. (2010) provided important evidence of imperfect transmission in natural settings.

Our study aimed to examine the transmission efficiency rates of pea aphid endosymbionts in natural settings with a focus on how symbiont community composition may influence transmission success. The results of our statistical analysis support the idea that vertical transmission efficiency may be influenced by the microbial community dynamics in pea aphids for at least some facultative symbionts in the pea aphid system.

Previous work done by Smith (2015), provided insight into which symbiont pairings might promote successful transmission rates. We considered the symbiont pairings Smith found more or less often than predicted by chance when developing sampling bins for our statistical analysis. Of particular importance was the *Rickettsiella-Serratia* pairing, which Smith's group found more often than predicted in 13 of the sample populations. A comparison of *Rickettsiella* and *Serratia* transmission efficiency revealed a difference in transmission success when these symbionts were found alone or with other symbionts outside of the *Rickettsiella-Serratia* co-infection. The highest vertical transmission efficiency occurred in the group of aphids infected with *Rickettsiella-Serratia* alone.

These results coupled with prior discoveries of pea aphid symbiont defense raise the possibility that the structure of microbial communities may be a predictor of vertical transmission efficiency. The *Rickettsiella-Serratia* symbiont pairing may be beneficial in multiple context-dependent situations. *Rickettsiella* is an anti-pathogen symbiont that also has the potential to turn red aphids green, perhaps allowing them to be less of a target for predators like ladybird beetles, which seem to be visually oriented toward red morphs a cryptic defense against predators such as ladybird beetles (Losey *et al*. 1997). *Serratia* may be able to confer low levels of resistance to parasitoids (Oliver *et al*. 2003) while also protecting aphids from the effects of high temperatures (Russell & Moran, 2006). Hence, the *Rickettsiella-Serratia* pairing may be selected for in nature as it provides a wide range of fitness benefits.

The presence of Spiroplasma appears to be associated with imperfect transmission of the *Rickettsiella-Serratia* pairing in nature. In our study, the transmission rates of *Rickettsiella* and *Serratia* went down by about 44.43% and 41.13%, respectively, when *Spiroplasma* was present. We also saw a decrease in these symbionts' transmission rates when two or more additional symbionts were cohabiting with the *Rickettsiella-Serratia* pairing. This suggests that the number of symbionts present within a single host organism can have an effect on transmission efficiency.

Understanding how microbial community structures may influence vertical transmission rates may be an important step in improving our ability to make predictions about symbiont frequencies in nature. Inherited, defensive symbionts have been found in a variety of plants and invertebrates including organisms that have economic, medical or agricultural importance.

Host-symbiont relationships that have the ability to alter organisms' diets can have agriculturally important implications. For example, the western corn rootworm is a major corn pest that has been controlled via annual rotation between corn and non-host soybean. Since the rootworm can traditionally only feed on corn, rotating the crops used to be very effective in reducing population sizes of these pests without having to use pesticides. Over time, however, this practice has selected for a "rotation-resistant" variant, which most likely evolved through a change in microbial community structure of the gut bacteria. Chu *et al*. (2013) compared the survival rates of the two types – the wild-type and the rotation-resistant. Their study concluded that the type with the altered microbial community has an advantage on soybean, which makes it a more of a threat to crops. Gut bacteria have also been shown to aid invasive species in adapting to feed on new crops. For example, *Megacopta cribraria* stinkbugs in the United States are originally from Asia and were previously known to feed only on kudzu. However, due to differential selection for genes related to nutrient provisioning, some of these stinkbugs are now able to feed on soybean, inflicting serious damage on soy crops (Brown *et al*. 2014). Both of these findings demonstrate that gut bacteria can help to facilitate rapid adaptation of insects in managed ecosystems, which may have major impacts on agricultural techniques and strategies.

Another important symbiotic relationship exists between mosquitoes and a symbiont called *Wolbachia*. The presence of *Wolbachia* in the mosquito can actually suppress the dengue virus in mosquitoes along with other viruses and malaria parasites (Eleftherianos *et al*. 2013). As an alternative to controlling mosquito populations using harmful chemicals, the NEA has been rearing *Wolbachia*-carrying mosquitoes and

releasing them into the wild in an attempt to reduce the prevalence of this virus. It is important to note here that this process of injecting *Wolbachia* in mosquitoes is not useful unless *Wolbachia* from the introduced mosquitoes gets transmitted from one generation to the next and it becomes established in natural populations. Studies aimed at examining transmission rates have found high rates of transmission in the lab, but less is known about transmission efficiency in natural settings. The patterns of natural symbiont transmission dynamics that we have uncovered in pea aphids could potentially support the work that has been done on agriculturally and medically important organisms. Our work may also aid in understanding the connection between ecology, biology, and evolutionary genetics.

Further Analysis

Initial examination of these data provides evidence that microbial community structure may influence vertical transmission rates. However, the exact intraspecific interactions that cause these shifts in transmission efficiency remain largely unknown. As symbiont density seems to impact transmission efficiency, it may be useful to utilize quantitative PCR techniques to analyze both the naturally occurring densities of symbionts within aphids and shifts in these densities caused by the presence or absence of additional symbionts. For instance, to determine whether the high transmission success rate of *Rickettsiella-Serratia* pairing could be related to the symbionts' effects on each other's densities, one could measure the average *Rickettsiella* density within a pea aphid clone that does not contain *Serratia*, and then inject this same clone with *Serratia* and

reevaluate *Rickettsiella* density. Potentially negative effects of *Spiroplasma* on *Rickettsiella* density could also be investigated in a similar fashion. A constant rise or fall in symbiont density across many replicates would provide evidence for potential symbiont cooperation or conflict within the aphid microbiome.

The location of the facultative symbionts within the host may also affect their density and transmission efficiency (Su *et al*. 2014). The location of secondary symbionts within a host can vary. They have been detected in locations such as Malpighian tubules (Bution et al, 2008), hemolymph (Braquart-Varnier et al, 2008), reproductive organs (Frydman, 2006), and salivary glands (Macaluso, 2008). The location of the symbionts may be an important factor involved in successful transmission. For instance, *Rickettsiella* and *Serratia* may localize in similar regions within the aphid allowing them to benefit from each other's presence. There are many examples of co-symbionts evolving within a host population such that the symbionts can no longer live as free-living organisms and become obligate for the host. In these types of systems, each symbiont typically provides the host with different benefits. Wu *et al*. (2006) found sharpshooters' dual bacterial symbionts, *Sulcia* and *Baumannia*, play complementary, non-overlapping roles: *Sulica* provides the host with amino acids and *Baumannia* provides the host with vitamins. Even more interesting is that the genomes of *Baumannia* and *Sulica* have been significantly reduced, which means they may now rely on each other for important nutrients. It is possible that symbionts localized in similar locations within the pea aphid could be developing these types of relationships where they support each other by sharing nutrients.

Location could also have a negative impact on transmission. An example of an antagontistic relationship between symbionts can be found in *Anopheles* mosquitoes. In these mosquitoes, *Wolbachia* are outcompeted by resident *Asaia* symbionts explaining *Wolbachia's* absence. It is possible that *Spiroplasma* is having a similar effect on other symbionts within the pea aphis system.

Cooperative and competitive relationships between symbionts in pea aphids could be investigated using fluorescence in situ hybridization (FISH) microscopy to determine the relative locations of the symbionts within the aphid. It is probable that symbionts living in close proximity impact each other's life history traits in either advantageous or antagonistic ways. Knowing their relative locations within an aphid could be useful for understanding how microbial community structures affect transmission. Furthermore, understanding how the acquisition of new symbionts may alter the locations of the original symbiont inhabitants would be informative because a shift in location may affect transmission.

Environmental factors can also influence the densities and potentially the locations of symbionts within an aphid. To rule out the alternative possibility that our results interpreted a drop in density as inefficient transmission, it is important to track symbiont presence in targeted lines for more than a single generation beyond the F_0 mother. The potential for missed symbiont detection caused by low symbiont density was a valid concern throughout this work as it could be mistaken for transmission failure. In our first year of this study, several of the field-caught lines were maintained in the lab at ideal conditions for ten generational cycles. This allowed the symbiont densities to reach an equilibrium. Within these lines, we did not find any evidence of symbionts that

appeared to be lost returning in later generations. It may be useful to repeat this investigation for a wider range of symbiont infections and clonal backgrounds to confirm symbiont loss and distinguish it from a shift in symbiont density.

In a significant fraction of the aphids used in this research, a symbiont present in one or more offspring was not present in the offspring's respective F_0 mother. We are confident given F_0-F_1 matches in our microsatellite genotyping results that aphid contamination did not occur for many of these instances. Negative controls also allowed us to rule out contamination during the extraction and the PCR stages. Therefore, there is most likely an ecological reason for what seem like symbiont gains within a clonal line. One possibility for these discrepancies is the symbiont in the F_1's was actually present in the F_0's, just at levels below the threshold of PCR detection. Another possibility is that the F_1 aphids acquired the additional symbiont through horizontal transmission.

The frequency of horizontal transmission in natural environments and the impact of horizontal transmission on microbial community dynamics could be important factors related to the likelihood of symbionts reaching fixation within a population. There have been some studies that have provided evidence for horizontal transfer (Chen & Purcell 1997; Darby *et al*. 2001; Sandström *et al*. 2001). However, not much is known about the extent to which horizontal transfer shapes secondary symbiont distributions.

Within the pea aphid system, horizontal transmission is currently thought to be much less frequent than vertical transmission and is not typically regarded as a major contributor to symbiont infections. One way to investigate the frequency of horizontal transmission would be to introduce lab-reared aphids with known infections into the field. The rate at which these aphids acquire symbionts would suggest potential rates of

horizontal transmission. Understanding the role horizontal transmission plays in the ecology and evolution of host-symbiont interactions may help us make useful predictions about the frequencies of defensive, inherited symbionts in natural settings.

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Appendix

Table S1. Primers and conditions for diagnostic PCR and sequencing. A) Primers used for diagnostic PCR and preparing sequence submissions. B) Thermocycling conditions.

A)

Diagnostic PCR Primer Pairs		
Buchnera aphidicola	BuchneraF: CTGTTGCCAGCCAGCGG TTCGG EcoliR: CCCCTACGGTAACCTTG TTACG	Leonardo and Muiru 2003
Hamiltonella defensa	10F: AGT TTG ATC ATG GCT CAG ATT G T419R:AAA TGG TAT TSG CAT TTA TCG	Sandstrom et al. 2001, Ferrari et al. 2012
Regiella insecticola	10F: AGT TTG ATC ATG GCT CAG ATT G Reg1292R: ACT TTA TGA GGT TCG CTT ACG	Sandstrom et al. 2001,Smith et al. 2015
Serratia symbiotica	10F: AGT TTG ATC ATG GCT CAG ATT G R443R: CTTCTGCGAGTAACGTC AATG	Sandstrom et al. 2001, Ferrari et al. 2012
X-type	10F: AGT TTG ATC ATG GCT CAG ATT G X420R: GCAACACTCTTTGCATT GCT	Sandstrom et al. 2001, Ferrari et al. 2012
Rickettsiella	RCL16S211F: GGG CCT TGC GCT CTA GGT RCL16S470R: TGG GTA CCG TCA CAG TAA TCG A	Tsuchida et al. 2010
Spiroplasma	9Fa: GAGTTTGATCITIGCTCA G Spi16SR: ATCATCAACCCTGCCTT TGG	Russell et al. 2009, McLean et al. 2011
Rickettsia	16SA1F: AGAGTTTGATCMTGGCT CAG RickR2: TCCACGTCACCGTCTTG C	Fukatsu and Nikoh 1998, Sakurai et al. 2005

Table S1 (continued)
B)

Buchnera aphidicola: 94◦C for 7 minutes; 30 cycles of 94◦C for 30 s., 62◦C for 1 min., 72◦C for 1.5 min; and 72◦C for 7 min.
Hamiltonella defensa, Regiella insecticola and X-type: 94◦C for 2 minutes; 9 cycles of 94◦C for 1 min., 65◦C for 1 min. decreasing by 1◦C each cycle, 72◦C for 2 min.; 25 cycles of 94◦C for 1 min., 55◦C for 1 min. and 72◦C for 2 min.; and 72◦C for 6 min.
Serratia symbiotica: 95◦C for 5 minutes; 40 cycles of 95◦C for 30s, 66◦C for 30s, 72◦C for 30s; and 72◦C for 2 min.
Rickettsiella: 95◦C for 4 minutes; 40 cycles of 95◦C for 30s, 58◦C for 30s, 72◦C for 30s; and 72◦C for 2 min.
Spiroplasma: 95◦C for 1 minute; 35 cycles of 95◦C for 1 min., 56◦C for 15s, 72◦C for 20s; and 72◦C for 2 min.
Rickettsia: 95◦C for 2 minutes; 12 cycles of 95◦C for 15s, 56◦C for 15s, decreasing by 1◦C each cycle, 72◦C for 30s; 35 cycles of 95◦C for 15s, 46◦C for 15s, 72◦C for 30s; and 72◦C for 1 min.

Vita

Danielle Irene Rock was born in Upper Darby, PA on December 6th, 1986. She attended Great Valley High School (Malvern, PA) and upon graduation was accepted into Pennsylvania State University (State College, PA) where she earned an undergraduate degree in mathematics with the teaching option. Since graduating in 2009, she has worked as a secondary mathematics teacher at Strath Haven HS and Germantown Academy (GA). In 2013, she began her fieldwork for her MS in Environmental Science with Dr. Jacob Russell. Her work is focused on the role symbiotic relationships play in evolutionary trends. She studied the transmission dynamics of symbionts in pea aphids under natural conditions, and has researched aphids with naturally occurring bacterial infections, especially aphids with multiple infections through field sampling, lab rearing, and extensive PCR screening. Danielle has also facilitated a lab in the Environmental Science courses at GA. She served as the liaison between the Russell Lab at Drexel and the Environmental Science teachers at GA during the completion of these laboratory experiments.